Scanning Electron Microscopy

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SCANNING electron microscopy is an ideal tool for description of microstructure in taxonomic studies. The scanning electron microscope (SEM) provides a surface image characterized by high resolution and depth of field and a three-dimensional quality unavailable with other techniques. In many cases this allows one to objectively describe microstructure where only subjective descriptions were available in the past. It is the purpose of this contribution to describe the techniques and use of scanning electron microscopy and its application to systematic investigations of fish eggs and larvae.

The SEM has been used in a wide variety of systematic and evolutionary investigations. With available magnifications from 10 to greater than 100,000 times, the SEM covers the range from dissecting and compound light microscopy to transmission electron microscopes. It has thus been immensely important to progress in classification in the study of micropaleontology, botany, insects and mites, and a wide variety of microorganisms, among other taxa (Heywood, 1971; Kormandy, 1975). Taxonomic applications of the SEM to fishes have been more limited. Several studies have used the SEM for studies of morphology, including epidermis, gill tissue, optic capsules, eggs, sperm, and embryos of fishes (Dobbs, 1974, 1975).

Microstructural analysis of otoliths of fishes with the SEM is now common (Pannella, 1980). For early life history stages, the most frequent use in identification and classification has been with the egg stage. The chorion, or external membrane, of many species is variously ornamented with filaments, spines, patterns of ridges, loops, blebs, and pustules (Ahlstrom and Moser, 1980; Robertson, 1981; Matarese and Sandknop, this volume). These ornamentations and the ultrastructure of the chorion are species-specific (Ivankov and Kurdyayeva, 1973; Lonning, 1972). While many of these structures may be easily visualized with light microscopy (Hubbs and Kampa, 1946; Kovalevskaya, 1982), the SEM often provides the best means of adequately describing structures which are very small or transparent under the light microscope. The egg chorion of *Maurolicus muelleri*, for example, was described as "drawn up into hexagonally arranged points," by Robertson (1976) based upon light microscopy but as "drawn up into hexagonal ridges . . . and slightly raised at the point of intersection" under the SEM (Robertson, 1981). Similarly, Boyd and Simmonds (1974), among others, suggested that the chorion of southern populations of *Fundulus heteroclitus* lacked fibrils using light microscopy, whereas the SEM showed the presence of numerous short and thin fibrils (Brummett and Dumont, 1981). Thus for purposes of classification, the SEM allows visualization of surface structures that are difficult to describe with light microscopy.

Methodology

Preparation of biological material for examination under the SEM is concerned with preservation, dehydration, and coating with a conductive material. Fixation of labile biological specimens is necessary because removal of water during the stages of dehydration may result in collapse of cells and other artifacts. Depending upon the method of fixation and dehydration, the artifacts can range from shrinkage to collapse or fracture of the structures to be observed. It is preferable to begin with fresh, live material. For eggs this requires either laboratory spawning or abundant eggs from the field which can be reliably collected. For larvae at different stages, it is difficult without laboratory rearing facilities. Results with formalin-fixed material from plankton collections will generally be satisfactory for lower magnification analysis of surface morphology, but may not reflect the quality of freshly prepared material.

Fresh material should be fixed for electron microscopy. Larval stages may first be relaxed in anesthetant solution (such as MS-222). Initial fixatives for both eggs and larvae are generally based upon glutaraldehyde, with concentrations ranging from 0.5 to 4.0%; lower concentrations are typically followed by post-fixation. A fixative which I have found acceptable is that from Dobbs (1974) as follows: 70% glutaraldehyde—2.0 ml, flounder saline—34 ml, and distilled water—34 ml. The flounder saline follows Forster and Hong (1958) and contains the following (in grams per liter): NaCl, 7.890; KCl, 0.186; $CaCl_2$, 0.167; $MgCl_2 \cdot 6H_2O$, 0.203; $NaH_2PO_4 \cdot H_2O$, 0.069; $NaHCO_3$, 0.84. The fixative has a final osmolarity of 380 mOsm/l. Fixation should be for 24 hours. Other authors provide several other fixatives. One suggested by Stehr and Hawkes (1979), while more difficult to prepare, is also useful should transmission electron microscopy be desired for the same material. Post-fixation in osmium tetroxide is recommended by several authors as a means of hardening particularly soft tissues. Generally, 1–2% osmium tetroxide in buffered saline is used. I have found this unnecessary with fish eggs and larvae, as suggested by Dobbs (1974) and Stehr and Hawkes (1979). It may be considered, however, if collapse is a problem. Lonning and Hagstrom (1975) suggested that egg chorions not post-fixed would rupture under the electron beam; I have not noticed this.

It is the process of dehydration where the greatest artifacts are likely to occur. With larvae, shrinkage of tissue may occur, while eggs may suffer complete collapse. On larger eggs, puncturing the chorion with a sharpened dissecting needle may facilitate transfer of fluids and prevent this collapse (Stehr and Hawkes, 1979).

Removal of water from the tissues is prerequisite to coating and observation, which are both conducted under high vacuum. Two methods are available, freeze drying and critical point drying. For freeze drying, unfixed fresh material may be used. Fixed material should first be rinsed with distilled water to remove salts, and then plunged with little adhering water into liquid nitrogen. Damage here may result from formation of ice crystals if freezing rate is too slow, but this is typically not a problem with small eggs and larvae in liquid nitrogen. Boyde and Wood (1969) recommend using 20 ml chloroform per liter of distilled water to increase nucleation rates and decrease ice crystal formation. After freezing, the material is immediately

introduced into the freeze dryer, where water sublimes, leaving the specimen dry and intact. Critical point drying, on the other hand, requires dehydration through a graded series of alcohols (20% for 24 h, then 10–20 min each in 50%, 70%, 80%, 90%, 95%, and two changes of absolute ethanol). The ethanol is then replaced with either freon or acetone depending on whether freon or carbon dioxide critical point dryers are used. The steps of dehydration and transfer can be done in small specimen holders to minimize handling and possible surface damage. After dehydration, specimens must be mounted on SEM studs with any of several available adhesives and tapes. The dried specimens are particularly delicate and should be handled with a small camel-hair brush to avoid damage to the surface. They are then oriented onto the stud under a dissecting microscope. Before coating, no further preparation is necessary with larvae, but eggs have only a small area of electrical contact with the stud. It is therefore advisable to use a conductive adhesive (such as silver paint) to make a more complete electrical connection and prevent charging, which decreases image quality. This paint should be allowed to become tacky prior to positioning the eggs, or it may cover portions of the egg itself. Finally, specimens are coated with a thin conductive layer, typically of gold or gold-palladium, by either vacuum evaporation or ion sputtering, prior to viewing on the SEM. At most facilities, trained SEM technicians are available; their advice and assistance are invaluable and should be sought.

Results and Discussion

Shrinkage and other artifacts will vary depending upon the type of material, preservation, and method of dehydration. For fresh material preserved in a mixture of formalin, glutaraldehyde, and acrolein, Stehr and Hawkes (1979) observed a shrinkage of approximately 10% in the eggs of *Platichthys stellatus* and *Oncorhynchus gorbuscha*; the latter had been punctured prior to dehydration. In the present study, eggs of *Maurolicus muelleri* initially preserved in 5% buffered formalin showed varying degrees of shrinkage and collapse depending upon subsequent treatment. The least shrinkage (12%, Fig. 18B) was noted in material which was freeze dried, whereas post-fixation and dehydration through freon 113 associated with critical point drying resulted in shrinkage of up to 67% of the original diameter (Fig. 18D). Eggs of this species show a hexagonal sculpturing; under the light microscope the sculpturing is hyaline and difficult to interpret (Fig. 18A). Eggs prepared by freeze drying clearly show the surface sculpturing; note particularly the ridges, which are more clearly defined (Fig. 18B). For comparison, an egg which had partially collapsed during dehydration is shown (Fig. 18D). The obvious differences in shrinkage point out the importance of specifying method, initial size, and shrinkage values, particularly for comparative or taxonomic studies.

Eggs from other species are shown to give an idea of the range of chorion structures which may be observed. The hexagonal pattern on *M. muelleri* overlies a highly porous surface structure

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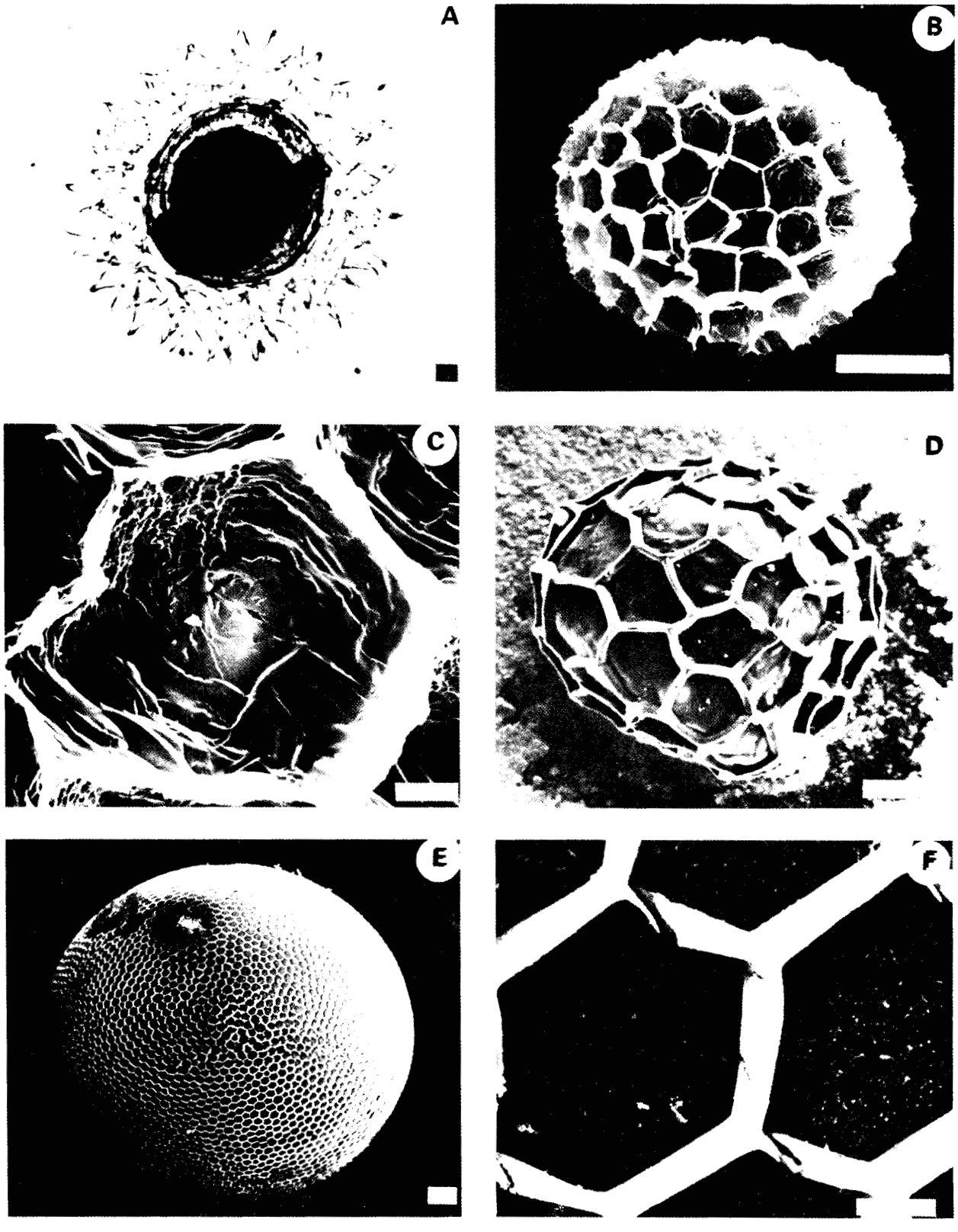
Fig. 18. (A) Egg of *Maurolicus muelleri* from off South Africa taken under the compound light microscope with transmitted, polarized light. Note the emphasis of the points on the hyaline chorion, which represent the intersections of ridges. Bar = 100 μm. (B) Egg of *M. muelleri* under the scanning electron microscope. Note the areas between what one would interpret as points on Figure 18A, which are now seen as polygonal facets or ridges. Bar = 500 μm. (C) Individual facet of the egg of *M. muelleri*. Note the porous and diaphanous nature of the egg surface. Bar = 50 μm. (D) Egg of *M. muelleri* post-fixed in osmium tetroxide and critical point dried. The shrinkage of this specimen is approximately 65%. Note the differences in morphology of the ridges and surface of the egg. Bar = 100 μm. (E) Egg of *Pleuronichthys coenosus*. The facets are relatively small by comparison with *M. muelleri* and the pattern units are more regularly hexagonal. Bar = 100 μm. (F) Detail of two hexagons from the egg of *P. coenosus*. Note the morphological differences between both the ridges and chorion surface as compared to *M. muelleri*. Bar = 10 μm.

Fig. 19. (A) Egg of *Atherinopsis californiensis*. The filaments are single, terminate in loose ends, and are distributed over the entire egg surface. Bar = 1,000 μm. (B) Egg of *Atherinops affinis*. The egg of this species is characterized by filaments which are looped, with no free ends (Curless, 1979). This differentiates it from the egg of *A. californiensis*, as do filament length, abundance, and basal morphology. Closed-loop filaments have also been noted in *Antennarius caudimaculatus* eggs by Pietsch and Grobecker (1980). Bar = 1,000 μm. (C) Chorion of *Paracallionymus costatus* collected off South Africa. The surface features are irregular and cover the entire egg surface. This differs from species of *Callionymus*, which have hexagonal patterns. Bar = 10 μm. (D) Chorion surface of *Mugil cephalus*. These structures are irregular and cover the entire egg surface. Note the superficial similarity to *Paracallionymus*. Bar = 10 μm. (E) Chorion surface of an advanced ovarian egg of *Coryphaenoides filifer*. Note that the surface "blebs" are arranged in hexagonal patterns and may be the precursors of a hexagonal pattern typical on eggs in this family. The pelagic egg of this species has not been described. Bar = 10 μm. (F) Chorion surface of an advanced ovarian egg of *Coryphaenoides acrolepis*. The hexagonal ridges are better developed than in Fig. 19E. There are holes under the ridges between the intersections, which might indicate that this species, whose egg is also undescribed, may have the hexagonal network supported on "stilts" as described for eggs of *Coelorhynchus* spp. (Robertson, 1981; Sanzo, 1933a). Bar = 10 μm.

Fig. 20. (A) Spines on the chorion surface of *Oxyporhamphus micropterus*. These are distributed over the entire surface of the egg. Bar = 100 μm. (B) Chorion surface from *Scomberesox saurus* collected off South Africa. The tufts are characterized by a relatively complex basal morphology and depending upon method of fixation, may resemble small bundles of hairs or, as here, simply coalesced tufts. Bar = 10 μm. (C) Micropyle and associated pores of the egg of *Lactoria diaphana* from the Eastern Tropical Pacific. The pores shown here are restricted to this region around the micropyle and appear to penetrate the outer layer of the chorion. Bar = 50 μm. (D) Secondary, smaller pit structures on the remainder of the egg of *Lactoria diaphana*. I refer to these depressions as "pits" because closer examination does not reveal penetration through any layer of the chorion, as opposed to the pores surrounding the micropyle in 20C. Bar = 1 μm. (E) Head region of a larval *Sebastes melanops* shortly after parturition. Polygonal epidermal cells may be noted on some parts of the body. Bar = 100 μm. (F) Epidermis on the dorsal surface, just posterior to the head, on an embryonic *S. melanops* approximately 28 days post fertilization. Note the distinct microridges and cell borders characteristic of developing teleost epidermis. Bar = 10 μm.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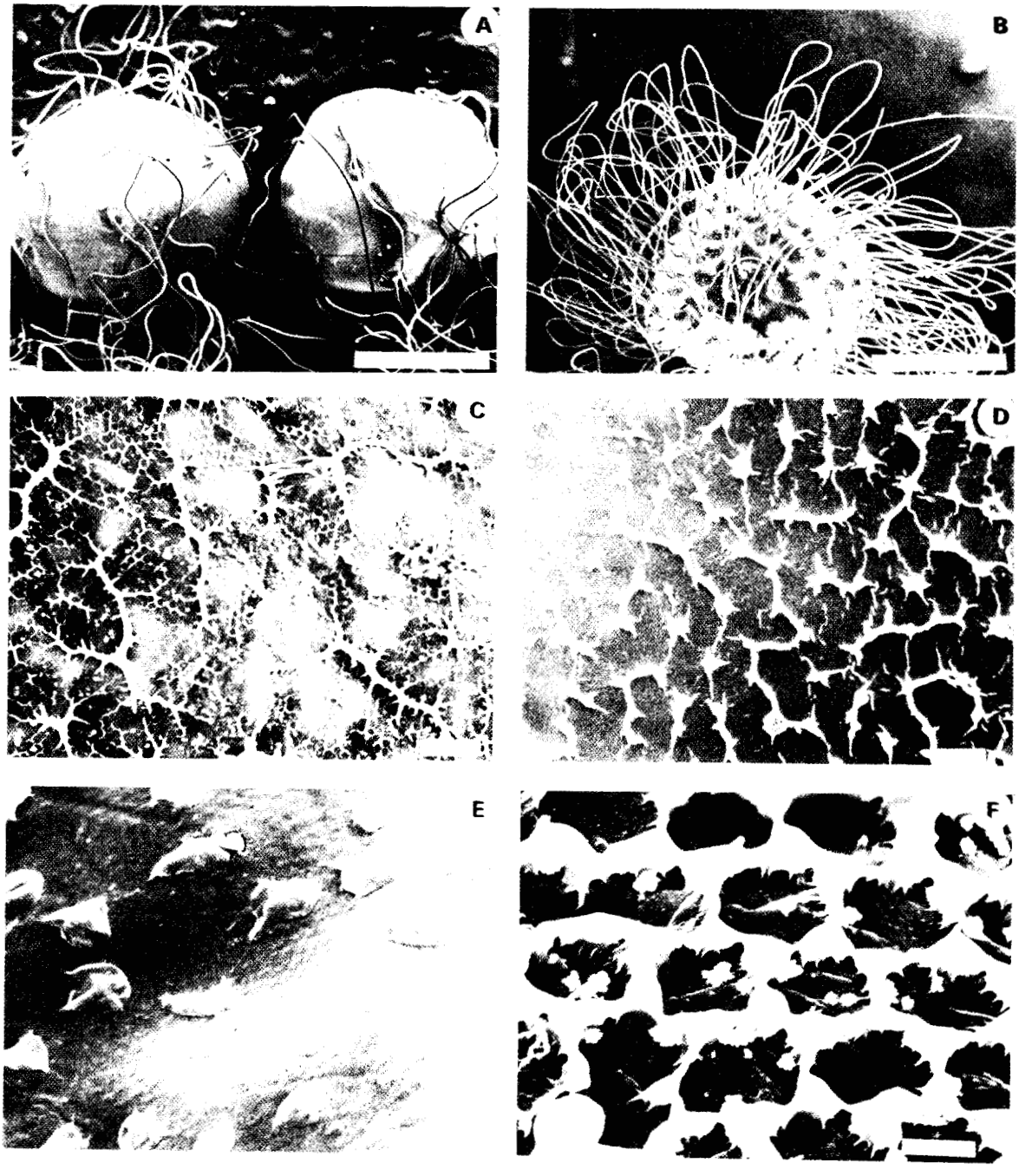
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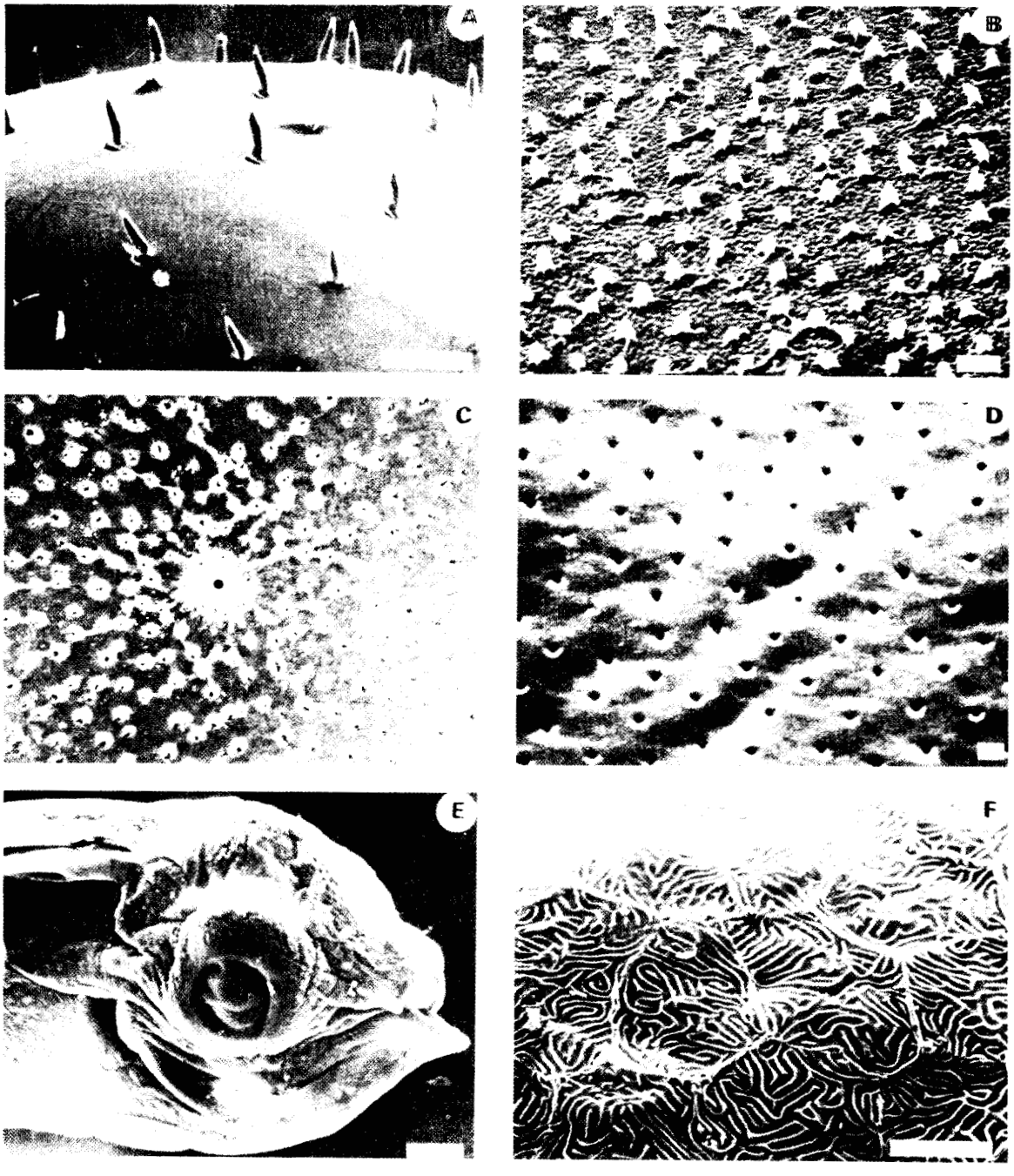
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(Fig. 18C) as compared to that of *Pleuronichthys coenosus* (Fig. 18E, F). Here, the hexagons are not only smaller, but the area within the facets does not appear porous. SEM was used for this species and its congeners for egg description by Sumida et al. (1979). It is interesting to note that these authors discussed the similarity in chorion structure of *Pleuronichthys* spp. with that of *Synodus lucioceps*. While there were slight differences in sizes of the polygons, the superficial similarity of chorion structure on these phylogenetically distant genera supports a functional role (Robertson, 1981) and independent derivation. In this instance, however, SEM was valuable for understanding and interpreting the differences between species and genera subsequently observed under the light microscope (Sumida et al., 1979). Similarly, Keevin et al. (1980) used chorion ornamentation to distinguish among genera of killifishes.

Other ornamentations include more random ridges (*Paracallionymus costatus,* Fig. 19C, and *Mugil cephalus,* Fig. 19D), filaments of varied length, diameter, and base morphology (*Atherinopsis californiensis* and *Atherinops affinis,* Fig. 19A, B; see also Hubbs and Kampa, 1946), tufts (*Scomberesox saurus,* Fig. 20B), spines (*Oxyporhamphus micropterus,* Fig. 20A), and pits and pores (*Lactoria diaphana,* Fig. 20C, D). In the callionymids, the small eggs of species of *Callionymus* have hexagonal sculpturing similar to that of *Pleuronichthys* (Fig. 18E). In *Paracallionymus costatus* (Fig. 19C), however, random ridges similar to those in *Mugil cephalus* are apparent.

Since chorion microstructure is formed by follicle cells during oogenesis (Sponaugle and Wourms, 1979; Stehr, 1979), patterns may also be discerned in ovarian eggs. The pelagic eggs of macrourids are poorly known but have been described for selected species by Sanzo (1933a), Robertson (1981), and Grigor'ev and Serebryakov (1981). For Pacific species of *Coryphaenoides,* pelagic eggs remain poorly known but apparently have hexagonal patterns as in other members of the genus; this is clearly shown in ovarian eggs near the maximum size observed by Stein and Pearcy (1982; Fig. 19E, F). Thus SEM of developing ovarian eggs may be used to discern differences which then aid in identification of eggs from plankton samples.

For larval stages, SEM has been used for the description of development of several surface structures, such as the olfactory organ (Elston et al., 1981) and lateral line neuromasts (Dobbs, 1974). For taxonomic studies, differentiation of fine-scale morphological differences, such as dentition or fine-scale spine serration, may be useful. Its most valuable use may therefore be for later larval development, since pigmentation and other characteristics in early larvae are better seen with conventional methods (Fig. 20E, F).

To conclude, SEM may serve as an adjuct to traditional methods in the description of fine structure in fish eggs and larvae. For high magnification, high resolution visualization of surface morphology, it remains the most effective tool available. Under lower magnifications, it may allow one to clearly visualize structures which are difficult to interpret using standard microscopical methods (Fig. 18A, B).

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